SIGNIFICANT CHANGES IN MICROBIAL COMMUNITY COMPOSITION IN THE GULF OF MEXICO "DEAD ZONE" OVER A DIEL CYCLE

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SIGNIFICANT CHANGES IN MICROBIAL COMMUNITY COMPOSITION IN THE GULF OF MEXICO "DEAD ZONE" OVER A DIEL CYCLE

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

SUMMARY

The structure and diversity of microbial communities associated with the oxygen minimum zone located on the Louisiana Shelf in the northern Gulf of Mexico deadzone was studied through amplicon analysis of the 16S rRNA gene. The oxygen minimum zone located on the Louisiana Shelf is a region of reduced oxygen concentrations, containing dynamic and diverse microbial communities that thrive under microaerophilic and anaerobic conditions. The Gulf of Mexico contains one of the largest zones of coastal hypoxia (region of reduced dissolved oxygen concentrations) which is dominated by complex microbial communities that contribute to marine biogeochemical cycling on a global scale. Here we used next-generation sequencing technology to track the microbial community at a single site over a day-night (diel) cycle. Two varying depths were used to collect seawater samples which were used for amplicon sequencing of the 16S rRNA gene (rDNA). By comparing our genetic data to coupled measurements of oxygen and nutrients, we determined how microbial community composition changes in response to day-night gradients and to environmental variation in oxygen and substrate availability.

CHAPTER 1

INTRODUCTION

Regions of reduced oxygen concentrations are common throughout the global ocean, containing dynamic and diverse microbial communities that thrive under microaerophilic or anaerobic conditions. The Gulf of Mexico houses one of the largest zones of coastal hypoxia (reduced concentrations of dissolved oxygen) in the shallow waters of the Louisiana Shelf bordering the outflows of the Mississippi and Atchafalaya Rivers. Marine hypoxic waters are generally prohibitive to higher trophic level organisms, and consequently are often termed "deadzones". However, marine deadzones, including those on the Louisiana Shelf, are dominated by complex-microbial communities that play important roles in marine biogeochemical cycling on a global scale. Microbial species in marine low-oxygen waters contribute to a major aspect of the carbon (C), nitrogen (N), and sulfur (S) cycles. For example, it has been measured that microorganisms in marine oxygen minimum zones (OMZs), notably the large OMZs occurring at midwater depth in the eastern Pacific Ocean, account for a major proportion (as much as 33%) of the loss of fixed nitrogen in the global ocean through the denitrifictation and anaerobic ammonia oxidation (annamox) processes and therefore play a major role in the global nitrogen budget (8). However, the knowledge of these processes occurring in seasonal OMZs such as the Gulf of Mexico deadzone is relatively limited, and it remains to be determined whether the same microbial groups and processes that occur in the permanent OMZs of the Pacific are also occurring in the coastal seasonal OMZs.

Based on recent metagenomic data, OMZ microbial communities show conserved patterns of community composition across various OMZs in the global ocean (19). However, despite an increasing understanding of microbial diversity and activity in open ocean OMZs, the microbial communities in the Louisiana shelf OMZ are relatively uncharacterized. The drivers of the microbial biodiversity in low-oxygen waters are understood at a general level. Nutrient influx, either from upwelling in the Pacific OMZs or from riverine outflow on the Louisiana Shelf, fuels high primary productivity in the photic zone, leading to a large influx of biomass. An active heterotrophic microbial community consumes this biomass as it sinks, depleting oxygen via aerobic respiration (2). As depth increases down the zone of oxygen depletion (oxycline), the microbial community shifts to dominance by microaerophilic or anaerobic members. Pelagic microbial community diversity has been shown to decrease with decreasing oxygen and this could possibly be a reflection of the loss of niche availability in the more environmentally constraining hypoxic layers (2). In the Pacific OMZs, in which the oxycline occurs at ~50-150 m depth, light levels are minimal, excluding photosynthesizing-microbes that interact with light (2). Therefore, in the core of the Pacific OMZs, niche availability has shaped the microbial community composition to represent a clustered unrelated community more similar in metabolic function than genetic relatedness. Indeed, it remains undetermined whether some of the same major microbial groups that are present in open ocean OMZs are also present and active in seasonally hypoxic waters, such as the Gulf of Mexico deadzone.

Unlike the permanent OMZs, the seasonal deadzone in the Gulf of Mexico occurs in shallow waters (<50m) on the continental shelf. Consequently, hypoxic layers in the

deadzone are also in the photic zone, suggesting the potential for diel-changes with the hypoxic zone community (14). Additionally, environmental conditions (e.g., oxygen, nutrient conditions) in the shallow deadzone may change rapidly in response to shifting oceanographic currents and storm activity (14). The deadzone therefore represents a valuable site for examining community microbial responses to temporal environmental shifts.

Spatial and temporal variation of microbial abundance, composition, and activity in response to diel (day-night) cycles and nonperiodic environmental disturbances are important factors in shaping the overall contribution OMZ microbial communities to bulk water column processes. Throughout the photic hours, when photosynthesis rates are high in surface waters, phytoplankton are highly active creating a large flux in surface biomass. This large amount of primary production should correlate with increased activity and abundance of heterotrophic bacteria in the surface waters. Indeed, based on a recent metagenomic study of microbial productivity in the eastern tropical North Pacific OMZ, data suggests that heterotrophic activity was highest in or above the oxycline (13). As the sun sets, the relative activity of different community members may shift to favor a relative dominance of heterotrophic over autotrophic processes.

Due to the large number of microbial-mediated processes occurring in OMZs that contribute to both the global marine nitrogen and sulfur cycles, it is important to analyze these microbial communities to better understand the phylogenetic make-up as well as functional activity of key microbial species present in the water-column and to understand how these communities respond to changes in environmental conditions. Abundant and rare microbial species make up OMZ communities, with varying metabolic

activities and growth rates. Abundant microbial taxa are hypothesized to be more active with high growth rates, while rare taxa are hypothesized to be more dormant and with slow growth rates (3). However, other studies have shown rare microbial taxa that are highly active with high growth rates and abundant microbial taxa that have slow growth rates and streamlined genomes (3). Therefore, the relationship between microbial abundance and activity is unclear for specific microbial taxa and needs to be further investigated.

In many OMZs, including the large permanent OMZs in the eastern Pacific, the low-oxygen zone occurs below the photic zone, suggesting that microbial activity in this layer is unlikely to vary over a day-night cycle. Activity in these zones is mediated by a slow but steady flux of sinking particulate carbon from the upper photic depths. Respiration of this carbon depletes oxygen and the community shifts towards one dominated by anaerobic or microaerophilic metabolism, including denitrification and the anaerobic oxidation of ammonia with nitrite (by *Planctomycetes* bacteria). The transition into this suboxic zone creates niches for other taxa, including ammonia oxidizing archaea such as *Thaumarchaeota,* which have been shown to thrive immediately above the anoxic zone of the permanent OMZs (13)

Deadzone microbial communities responding to environmental and diel shifts have potentially important roles in elemental cycling. Certain abundant and ubiquitous microbes belonging to the *Alphaproteobacteria* clade are thought to dominate prokaryotic communities in many pelagic environments and could possibly be highly abundant in OMZ surface waters (13). Also, ammonia oxidizing bacteria and ammonia oxidizing

archaea are thought to be ubiquitous in certain temperate pelagic environments leading to a potential abundance in OMZ surface waters.

Recent metagenomic analysis of nitrification gene abundance from our study site on the Louisiana Shelf (conducted in collaboration with Laura A. Bristow, University of Southern Denmark) showed strong temporal shifts in the abundance of the amoC gene over the course of a 2-day collection period. The amoC gene is involved in aerobic ammonia oxidation, which is common along the periphery of low-oxygen layers. Shifts in amoC suggests changes in the relative importance of aerobic nitrification surrounding the deadzone. Indeed, this is supported by recent evidence for high variation in nitrite oxidation rates at our study site (Bristow et al unpublished data).

The goal of our study is to examine changes in pelagic microbial community composition over a 2 day day-night cycle during summer 2012 on the Louisiana Shelf. During the summer months in 2012, the Louisiana Shelf was relatively oxygen rich compared to previous summers. Nonetheless, our sampling station captured a relatively wide range in oxygen and nutrient conditions during the 2-day sampling period. We determined microbial community composition (relative abundance of individual taxa) using amplicon analysis of the 16S rRNA gene (rDNA).

For this study, seawater samples were collected every 4 hours in the Gulf of Mexico deadzone at a single site at one mid-water depth (7 m) and one bottom-water depth (15 m) over a diel cycle. By collecting samples over short time scales, we can analyze how immediate shifting environmental conditions play a role in changing microbial community composition at a single hypoxic site. This allows for the potential recognition of a magnitude of biogeochemical cycling to change over time as well as

reveal how appropriate it is to infer the composition of a marine microbial community based on a single time-point sample versus multiple time-point samples at a single site.

The mid-water 7 m depth was characterized as the depth where the initial decline in oxygen concentration begins. The bottom-water 15 m depth represented the zone of maximum oxygen depletion, although, oxygen conditions were highly variable over our sampling time period. The seawater samples were subjected to DNA extraction and analyzed by amplicon sequencing on the Illumina Miseq Platform to assess the relative abundance of major groups of bacteria. By comparing our genetic data to coupled measurements of oxygen and nutrients, we can determine how microbial community composition changes in response to day-night gradients and to environmental variation in oxygen and substrate availability. This study can hope to shed light on a previously undescribed microbial community and to determine the similarity in seasonal coastal OMZs, such as the Gulf of Mexico deadzone, to large permanent, open ocean OMZs and to reveal whether or not similar microbial communities and metabolisms are existing in these two marine habitats.

CHAPTER 2

LITERATURE REVIEW

Marine Oxygen Minimum Zones and the Gulf of Mexico Dead Zone

Ocean waters with low oxygen saturation occur throughout the world. These zones, deemed oxygen-minimum zones (OMZs), occur in many deep water environments, but are also occurring more frequently in coastal regions due to anthropogenic affects (14). This is evident in the Gulf of Mexico, as it is home to one of the world's largest zones of coastal hypoxia. This OMZ is referred to as the "Dead Zone" and is located on the Louisiana/Texas continental shelf that borders the outflows of the Mississippi and Atchafalaya Rivers. This hypoxic zone is characterized as an area where the dissolved oxygen concentration drops to levels below 50μmol/kg as depth increases in the water column. Low dissolved oxygen concentrations in marine waters can kill higher level organisms, potentially altering ecosystem dynamics in one of the most economically important fisheries in the southeastern United States (8).

The Gulf of Mexico Dead Zone is a seasonal OMZ and is caused by the mixing of the fresh-water river outflow from the Mississippi and Atchafalaya Rivers with the warm coastal waters through regional water mass shifting patterns (14). This freshwater (low salinity) lens, which occurs over the shelf waters as a result of river input, causes an increase in nutrient loading in the marine surface waters as the river outflow is full of nitrogen and phosphorus compounds that originate from agriculture runoff, notably during the spring and summer months (8). The nitrogen and phosphorus derived from agricultural activities accumulates in the Mississippi River drainage basin and then enters the Gulf of Mexico. The excess nutrients lead to an increase in phytoplankton primary

production in the surface waters causing the accumulation of large algal blooms (2). As this flux in biomass sinks, microbial heterotrophs deeper in the water column decompose the organic matter leading to the depletion of the photosynthesized-derived oxygen (4). Oxygen concentrations are not resupplied in the water-column due to regional current patterns and stratification during the summer months. This generates a steep oxycline which acts as a physical barrier to higher level organisms and shapes microbial diversity in the water column.

Microbial Communities Present In OMZs

The Gulf of Mexico OMZ is dominated by diverse microbial communities that contribute to biogeochemical cycling on a global scale. Complex environmental factors, such as the interface between both riverine and marine waters and the shifting nutrient regimes, potentially drive microbial biodiversity in this zone in such a way as to represent a highly diverse and temporally dynamic microbial community. OMZs are represented by a steep geochemical gradient that leads to microbial spatial diversity within the water column shaped by oxygen concentration. While higher organisms cannot survive under hypoxic conditions, many microbes are well adapted to live in the absence of oxygen by using alternative electron acceptors. Thus, hypoxia causes a shift to an entirely microbe-dominated system.

Microbial-mediated activities in OMZs contribute to at least 33% of fixed nitrogen loss in the global ocean (2-4). Nutrient rich surface water fuels primary production which leads to the sinking of large amounts of organic matter. As heterotrophs deplete the available oxygen in the water column through the consumption of the surface-derived biomass, a dynamic nitrogen cycle develops in the anoxic conditions (2-4). In these zones, denitrification in which nitrate is fully reduced to N_2 gas occurs on a

relatively large scale. It is estimated that denitrification within OMZs accounts for the loss of as much as 275-400 Tg fixed N per year (8). Therefore, microbial-mediated processes in OMZs contribute to the formation of N_2 gas on a global scale and need to be accounted for. The anaerobic ammonia oxidation, or anammox, process in which ammonia is fully oxidized to N_2 gas may also be occurring in some OMZs, notably those in the eastern tropical Pacific (4). In this process, nitrite is used as the terminal oxidant to oxidize NH_4 and is thought to account for a large proportion of fixed nitrogen loss in the global ocean (8). This leads to a potential coupling between denitrification and annamox, as denitrification provides the nitrite used in the annamox process (8). However, overall, the knowledge of these processes occurring in seasonal hypoxic zones, such as the Shelf deadzone, is relatively limited and it remains to be determined whether the same microbial groups and metabolisms that mediate N cycling in the large anoxic OMZs of the Pacific are also active in the Gulf of Mexico deadzone.

As nitrogen cycling dominates the geochemistry of OMZs, it has been shown recently that a cryptic sulfur cycle also contributes to elemental cycling in the water column (4). Recent metagenomic data suggest the existence of both sulfide-oxidizing and sulfate-reducing bacteria that contribute to a cryptic sulfur cycle in the Pacific OMZs (4). The sulfur-oxidizing bacteria are chemoautotrophs that oxidize sulfide with nitrate as the electron acceptor to obtain energy for carbon fixation. Thus, their metabolism contributes directly to the N, S, and C cycles in these zones. This shows for the potential coupling between nitrifiers, annamox, and S-oxidizing bacteria pushing low oxygen waters to function as CO_2 sinks (4). However, these bacteria have been studied only in permanent OMZs, therefore, their importance in coastal OMZs is not yet understood.

The expansion and persistence of seasonal OMZs, such as the Dead Zone, appear to be a consequence of anthropogenic-related climate change and coastal eutrophication (13). The fact that the Gulf of Mexico OMZ has been shown to be expanding in size over the past half century, stresses the importance of defining this system and the ecological parameters that control it. Through the increase in use of agricultural fertilizers, which input large amounts of nitrogen and phosphorus into river runoff, the Gulf of Mexico OMZ is becoming more extreme with increasing cases of bottom-water hypoxia throughout the region (8). Oxygen depletion in the water column alters the structure and function of the microbial communities present which, in turn, alters the geochemical cycling as well as the energy flow from the microbial community to higher level organisms (13). This can lead to large amounts of environmental and financial devastation in one of the United States largest commercial fisheries.

Due to the diverse metabolic processes that occur in OMZs that contribute to geochemical cycling on a global scale, it is important to not only analyze the microbial community on a taxonomic level but also determine the activity of the players that make up that community. By sampling over a day-night cycle, it is possible to examine how these communities change in composition over short time scales in response to shifting environmental conditions. This not only reveals the potential for the magnitude of biogeochemical cycling to change over time, but also about how appropriate it is to infer the composition of marine microbial communities based on static snapshots (i.e. single time-point samples).

Recent advancements and lowering costs in genomic sequencing techniques now allow us to determine the taxonomic identity of thousands of marine microbiota in a

single analysis. The application of genomics to microbial oceanography has led to a dramatic increase in the understanding of marine ecological systems.

Chapter 3

Materials and Methods

Sample Collection

Seawater samples were collected during a July 2012 Gulf of Mexico cruise aboard the *R/V Cape Hatteras*. All oceanographic properties were obtained using a conductivity-temperature-depth (CTD) system mounted on a rosette. Water samples were collected every 4 hours over a 48 hour period via 20-L Niskin bottles deployed on a rosette system (7m, 15m). Ten liters of seawater was then transferred to a 10-L carboy and pre-filtered through a GF/A pre-filter (47mm, 1.6µm) and filtered onto Sterivex collection filters using a pump system. Pre-filters and Sterivex filters were then preserved with Lysis Buffer and stored in -40ºC freezer.

DNA Extraction and Isolation

Sterivex filters stored with Lysis Buffer until DNA extraction using a standard DNA extraction protocol was followed using a phenol:chloroform extraction from (Ganesh, et al. 2014). Briefly, cell were lysed by adding lysozyme directly to the Sterivex filter then sealing the ends and incubating for 45 min at 37°C. Proteinase K (1mg in 100 ml lysis buffer, with 100 ml 20% SDS) was added, and the Sterivex filters were sealed and further incubated for 2h at 55°C. The Lysate was removed and nucleic acids were extracted once with phenol:chloroform:isoamyl alcohol and once with choloroform:isoamyl alcohol (9). The aqueous phase was then concentrated by spin dialysis using centrifugal filters (9). Samples were then quantified using Nanodrop and stored at -240ºC until downstream processing.

PCR Amplification and Amplicon Generation

DNA samples were used for PCR amplification with universal primers targeting the V1-V3 region of the bacteria 16S rDNA gene. Primer sequences were adapted from (Caporaso, et al. 2012), and appended with sample-specific barcodes to enable multiplexed sequencing, and with adaptors specific to the Illumina sequencing platform for sequencing on the MiSeq Platform. PCR amplicon synthesis occurred with thermal cycling conditions established from the (Ganesh, et al. 2014) protocol which included: initial denaturation at 95º C (2min), followed by 30 cycles of denaturation at 95ºC (20 s), primer annealing at 50ºC (30 s) and primer extension at 72ºC.

Illumina Miseq Platform and Sequencing Analysis

Paired-end sequencing (250X250 bp) of PCR amplicons was conducted on the MiSeq platform (Stewart lab) according to (Caporaso et al. 2012). A control library was made from phiX174 during the sequencing runs. The phiX control was used to account for the limited sequence diversity among the 16S amplicons. The sequences were then subjected to quality filtering and analysis. All trimming, clustering, and classifications were performed in QIIME following the protocol form (9). The barcoded 16S data sets were de-multiplexed and subjected to quality filtering to remove low quality sequences using default parameters (minimum quality score =25, minimum sequence length = 200, no ambiguous bases allowed) (9). Sequences were then clustered into operation taxonomic units (OTUs) at 97% sequence similarity. The taxonomy was assigned to representative OTUs from each cluster using the Ribosomal Database Project classifier in QIIME, trained on the Greengenes database (9). The relative abundances of microbial OTUs were calculated as a percentage of the total number of QIIME-classified reads.

Chapter 4

Results

Station 6 Nutrient Profile

Samples were collected at a single site (29°5.983"N, 92°11.942W) on the Louisiana Shelf in the northern Gulf of Mexico. The lowest oxygen concentrations occurred within a narrow band (~2-3 m) immediately above the sediment-water interface (~15 m water depth). Oxygen concentrations were ~20 µmol/kg at the 15 m depth at the start of the collection and 78.53 µmol/kg ± 22.55 µmol/kg over the course of the 48-hour sampling period (Average of O_2 concentrations measured every 4 hours; Figure 1). The 7 m sampling depth marked the upper portion of the oxycline. Surface waters were characterized by a low-salinity cap due to fresh-water river outflow mixing with the coastal water. At the 7 m depth, the oxygen concentration averaged to be 156.19 µmol/kg ± 54.06 µmol/kg over the course of the sampling time period. Statistical analysis through a tradition t-test showed that the salinity concentrations (n=13, p=1.39E-05, t test) and oxygen concentrations (n=13, p=0.00032, t test) were significantly different ($p<0.05$) between the 7 m depth and 15 m depth across all time points (Figure 1).

Nitrite and nitrate concentrations increased with depth, peaking at 4 µM and 6 µM, respectively, at the 15 m. Ammonia oxidation rates were measured at ~100 nmol/day at the 7m depth and 500 nmol/day at the 15 m depth. Nitrite oxidation rates were measured at ~400 nmol/day at the 15 m depth (Rate data from L. Bristow, unpublished).

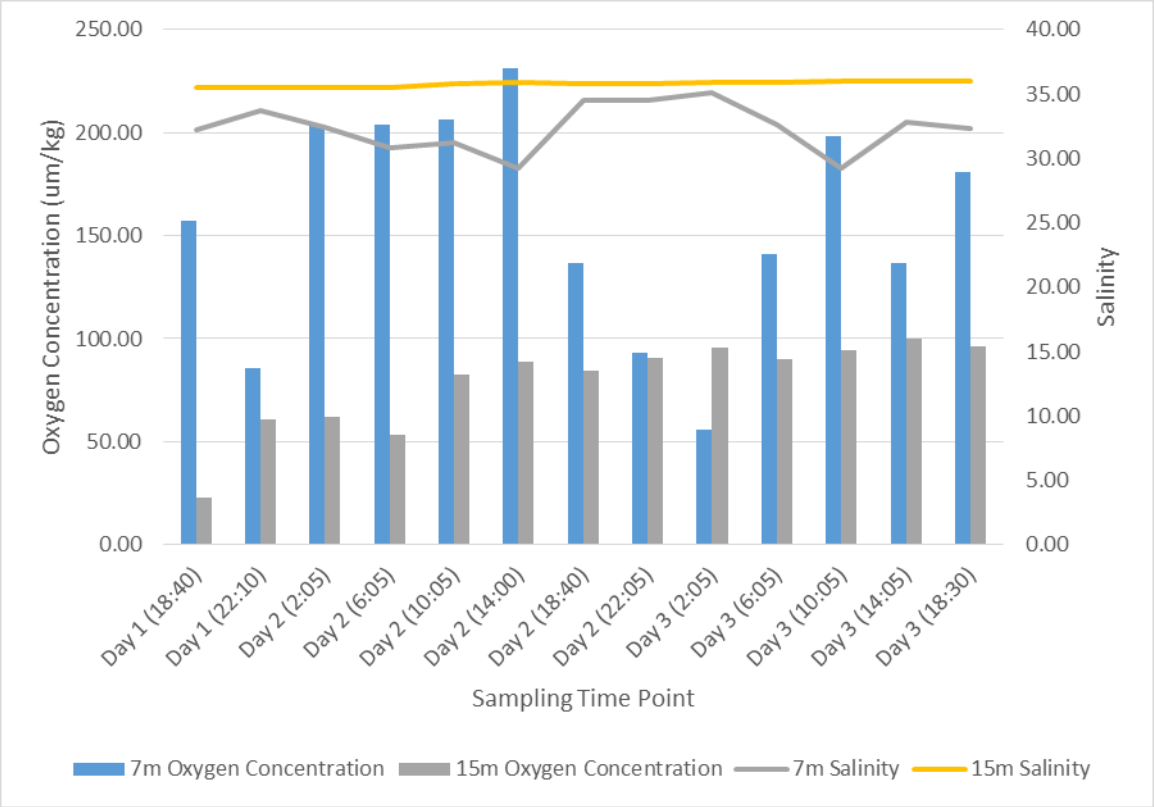

Figure 1: Station 6 Nutrient Profile. The lowest oxygen concentrations were measured at the 15 m depth with a 78.53 µmol/kg ± 22.55 µmol/kg average over the course of the sampling time period. At the 7 m depth, the oxygen concentration averaged to be 156.19 µmol/kg ± 54.06 µmol/kg. The 7 m depth showed a lowered, more variable salinity cap due to freshwater input into the system. Statistical analysis showed that the salinity concentrations (n=13, p=1.39E-05, t test) and oxygen concentrations (n=13, p=0.00032, t test) were significantly different ($p<0.05$) between the 7 m depth and 15 m depth across all time points.

Sequencing Analysis

Illumina Miseq sequencing of the 16S rRNA gene produced 4,978,789 barcoded, unmerged sequences representing 22 samples taken from the 2 depths (7 m, 15 m) over the 2 day period were considered for analysis.

Microbial Community Composition at the 7 m Depth

Community composition at the 7 m depth was analyzed for 10 seawater samples taken over the 3 day time period (Figure 2). We followed changes in the relative (proportional) abundance of individual microbial phyla (with *Proteobacteria* subdivided according to class) over time to explore changes in microbial community composition with respect to the ecology at that time point. When measuring across all time points, the majority of sequences at the 7 m depth were *Actinobacteria* (26%), followed by *Gammaproteobacteria* (16%), *Cyanobacteria* (16%), *Thaumarchaeota* (10%), and *Euryarchaeota* (10%).

Abundant phyla were described as those that comprise 1% or more of the community while rare phyla were described as those that comprise less than 1% of the community. This allowed separation of phyla present at this depth into categories of abundance based on criteria: abundant= OTUs>1% for all time points, mostly abundant= OTUS>1% for >50% of time points, cycling=OTUS>1% for <50% of time points, and rare=OTUs<1% for all time points. Of the 29 phyla present at the 7 m depth over the three day period, 6 were abundant all of the time and 3 were abundant at least 50% of the time (mostly abundant). Whereas 3 phyla cycled between abundant and rare (cycling, abundant <50% of the time), and 17 were always rare. The 6 abundant phyla at the 7 m depth were *Euryarchaeota*, *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Alphaproteobacteria*, and *Gammeproteobacteria*. Only 3 groups (mostly abundant: *Thaumarchaeota*, *Deltaproteobacteria*, and *SAR406*; cycling: *Chloroflexi*, *Verrucomicrobia*, and *ZB3*) were represented in both the mostly abundant fraction and cycling fraction respectively. The remaining 17 phyla represented the rare fraction of the community at the 7 m depth.

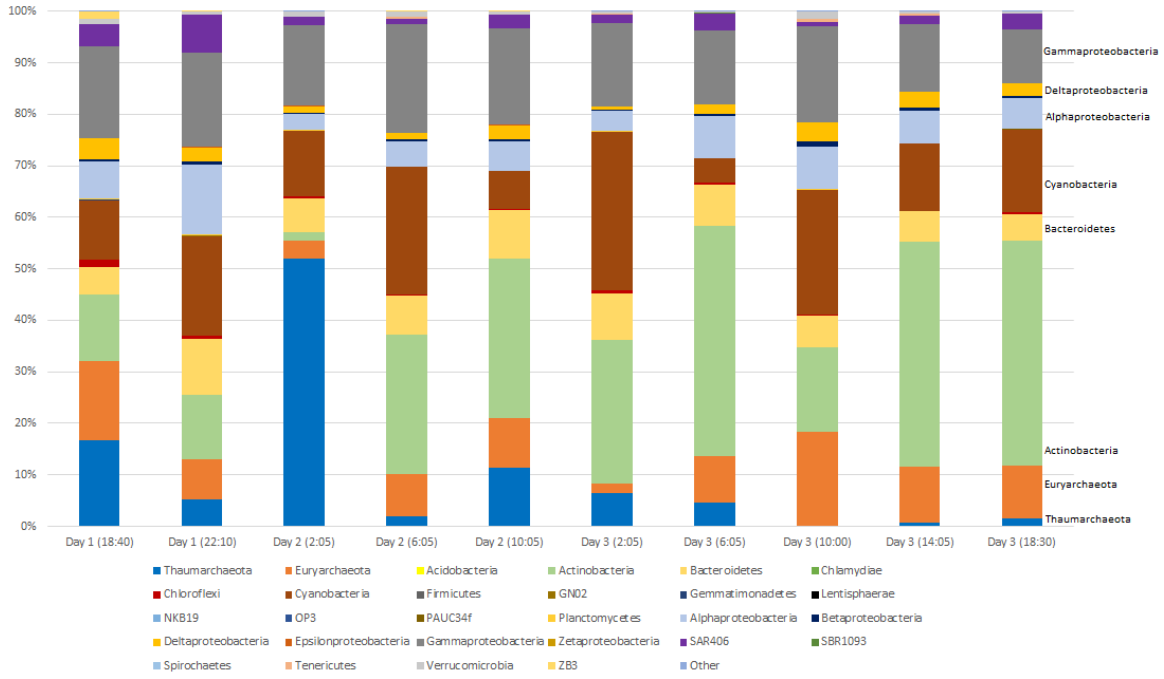

Figure 2: Microbial community composition at 7 m depth. 16S amplicon sequencing of 10 seawater samples taken from the 7 m depth. Individual taxa abundance was calculated by taking the successful 16S rDNA hit counts as a percentage of the total amount of reads at each time point. When measuring across all time points, the majority of sequences at the 7 m depth were *Actinobacteria*, followed by *Gammaproteobacteria*, Cyanobacteria, *Thaumarchaeota*, and *Euryarchaeota*.

Community Shifts in Response to Diel Cycle at the 7 m Depth

Microbial community shifts in response to the day-night cycle were examined by analyzing the relative abundances during the photic time points to the relative abundances during the night time points at the 7 m depth. Of the 29 major phyla present at this site, only 1 group (unclassified phylum *OP3*) was considered significantly different ($p<0.05$) using a standard t test with the relative abundances during photic hours compared to relative abundances during the night hours. However, when considering a p-value<0.1 to acknowledge significance, 7 groups were considered significantly different ($p<0.1$) when comparing the relative abundances during the photic time points to the relative abundances during the night time points at the 7 m depth. The 7 groups were represented

by *Acidobacteria* (n=5, p=0.063, t test), *Chlorofelxi* (n=5, p=0.051, t test), *OP3* (n=5, p=0.046, t test), *Epsilonproteobacteria* (n=5, p=0.092, t test), *Gammaproteobacteria* (n=5, p=0.085, t test), *Spirochaetes* (n=5, p=0.052, t test), and *Tenericutes* (n=5, p=0.051, t test) (Table 1).

OTUs	Photic Time Points Mean Abundance	Dark Time Points Mean Abundance	p-value	Observations
OP3	<1%	<1%	0.046*^	5
Acidobacteria	<1%	<1%	0.063*	5
Chloroflexi	<1%	<1%	0.051*	5
Epsilonproteobacteria	<1%	<1%	0.092*	5
Gammaproteobacteria	17.2%	15.7%	0.085*	5
Spirochaetes	<1%	<1%	0.052*	5
Tenericutes	<1%	<1%	0.051*	5

Table 1: Community shifts in response to diel cycle at the 7 m depth. Out of the 29 OTUs present at this site, only 1 group was considered significantly different ($p<0.05$)^ when comparing the relative abundances during the photic time points to the relative abundances during the night time points at the 7 m depth. The OTU in this category was identified to be *OP3* at the phylum level. However, when considering a p-value<0.1 to acknowledge significance, 7 groups were considered significantly different ($p<0.1$)*. The 7 groups were represented by *Acidobacteria*, *Chlorofelxi*, *OP3*, *Epsilonproteobacteria*, *Gammaproteobacteria*, *Spirochaetes*, and *Tenericutes*.

Microbial Community Composition at the 15 m Depth

Microbial community composition at the 15 m depth was analyzed by amplicon sequencing 16S rDNA in 12 seawater samples taken in cohort with the 7 m samples over the 2 day period (Figure 3). When averaging the abundances across all time points, the majority of the OTUs, when defined at the phylum level or subdivision level for Proteobacteria, were represented by *Gammaproteobacteria* (19%), and followed by *Cyanobacteria* (17%), *Actinobacteria* (15%), *Euryarchaeota* (12%), and *Alphaproteobacteria* (9%).

Phyla that were found at the 15 m depth were separated into 4 groups of abundance based on the same criteria used at the 7 m depth (abundant= OTUs>1% for all time points, mostly abundant= OTUS>1% for >50% of time points, cycling=OTUS>1% for <50% of time points, and rare=OTUs<1% for all time points). Of the 29 major phyla present at this depth, 9 were abundant across all time point whereas only 1 phyla was abundant more than 50% of the time (mostly abundant). Only 2 groups represented the cycling fraction of the community and the remaining 17 major phyla represented the rare fraction of the community. The 9 abundant phyla at the 15 m were represented by *Thaumarchaeota*, *Euryarchaeota*, *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Alphaproteobacteria*, *Deltaproteobacteria*, *Gammaproteobacteria*, and *SAR406.* The mostly abundant group was represented by *Verrucomicrobia*, while the cycling groups were represented by *Firmucutes* and *Betaproteobacteria*.

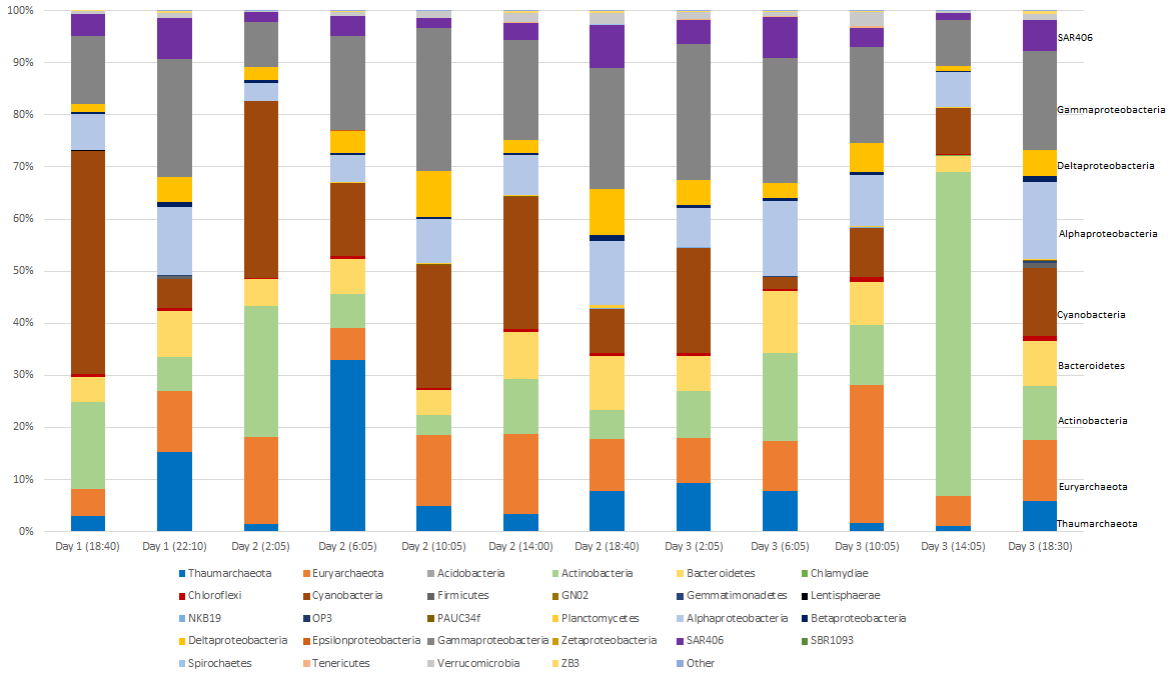

Figure 4: Microbial community composition at 15 m depth. 16S amplicon sequencing on 12 seawater samples taken from the 15 m depth. Relative abundance of individual taxa

measured by taking the number of successful hits out of the total number of sequences for that time point.

Community Shifts in Response to the Diel Cycle at the 15 m Depth

Microbial community shifts in response to the day-night cycle were also examined at the 15 m depth using the same statistical analysis (t test) used at the 7 m depth. Of the 29 OTUs present at this site, only 2 groups were considered significantly different ($p<0.05$) when comparing the relative abundances during the photic time points to the relative abundances during the night time points at the 15 m depth. The OTUs in this category were identified to be *Betaproteobacteria* and *Zetaproteobacteria* at the class level. However, when considering a p-value<0.1 to acknowledge significance, 6 groups were considered significantly different ($p<0.1$) when comparing the relative abundances during the photic time points to the relative abundances during the night time points at the 15 m depth. The 6 groups were represented by *Firmicutes* (n=6, p=0.098, t test), *NKB19* (n=6, p=0.082, t test), *Betaproteobacteria* (n=6, p=0.021, t test), *Zetaproteobacteria* (n=6, p=0.039, t test), *SAR406* (n=6, p=0.097, t test), and *ZB3* (n=6, p=0.078, t test) (Table 2).

OTUs	Photic Time Points Mean Abundance	Dark Time Points Mean Abundance	p-value	Observations
Betaproteobacteria	<1%	<1%	0.021*^	6
Zetaproteobacteria	<1%	<1%	0.039*^	6
Firmicutes	<1%	<1%	0.098^	6
NKB19	<1%	<1%	0.082^	6
SAR406	3.6%	5.5%	0.096^	6
ZB3	<1%	<1%	0.078^	6

Table 2: Community Shifts in Response to Diel Cycle at the 7 m Depth. Out of the 29 OTUs, defined at the phylum level, present at this site, 2 groups were considered significantly different ($p<0.05$)* when comparing the relative abundances during the photic time points to the relative abundances during the night time points at the 7 m depth. The OTUs in this category was identified to be *Betaproteobacteria* and *Zetaproteobacteria* at the class level. However, when considering a p-value<0.1 to

acknowledge significance, 6 groups were considered significantly different ($p<0.1$)^. The 6 groups were represented by *Betaproteobacteria*, *Zetaproteobacteria*, *Firmicutes*, *NKB19*, *SAR406*, and *ZB3*.

Overall Microbial Community Composition at Station 6:

Overall microbial community composition at station 6 was analyzed by combining the relative abundances of each group at each individual depth across all time points. The two total abundances were then combined and averaged to produce a compiled OTU average across both depths across all time points (Figure 4). Once both depths were combined, the majority of sequences were *Actinobacteria* (20%), *Gammaproteobacteria* (18%), *Cyanobacteria* (17%), *Euryarchaeota* (11%), *Thaumarchaeota* (9%) and *Alphaproteobacteria* (8%).

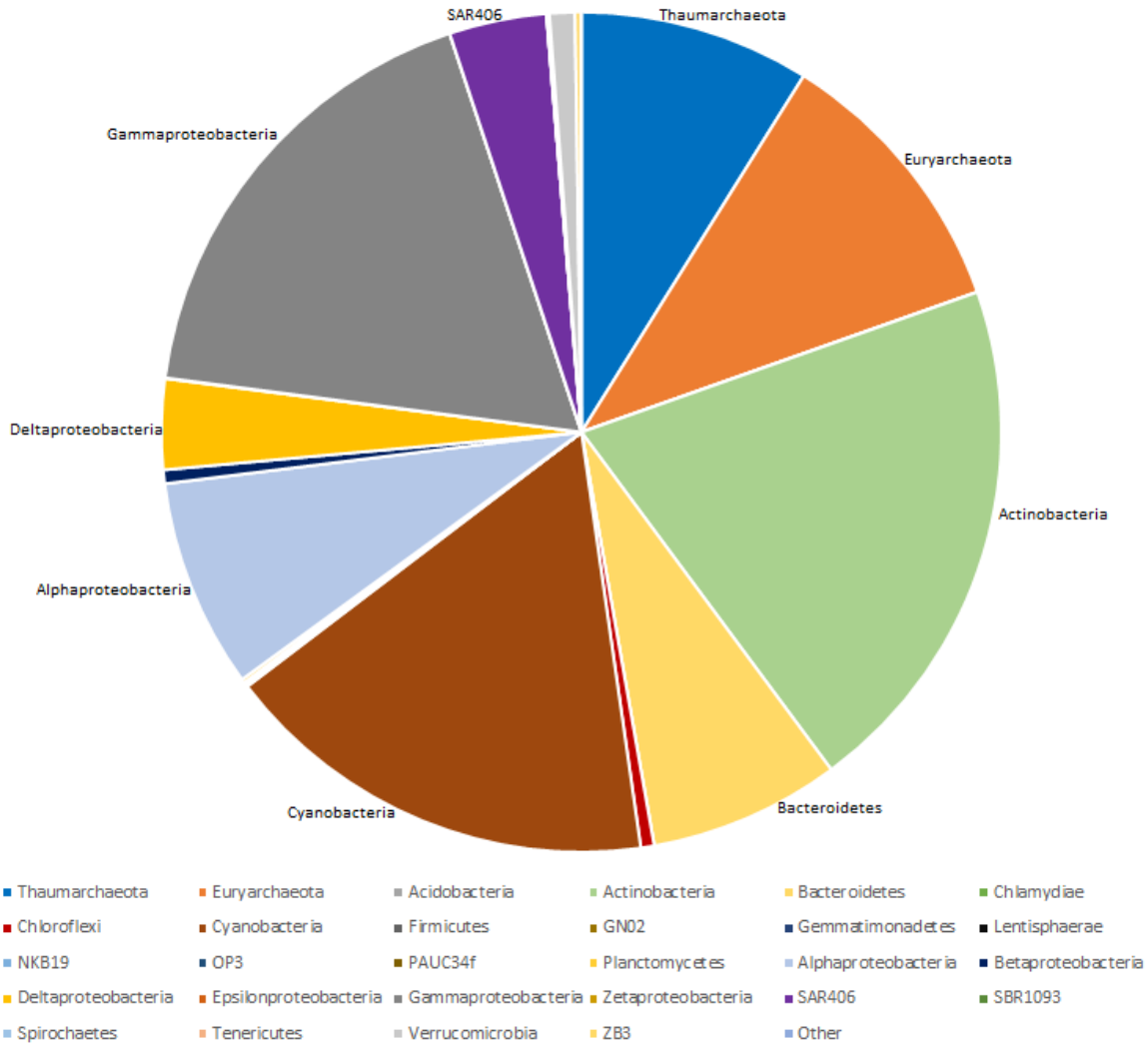

Figure 4: Compiled Average Abundance between both Depths over the 3 Day Sampling Period. The majority of sequences were Actinobacteria (20.27%), Gammaproteobacteria (17.85%), Cyanobacteria (16.91%), Euryarchaeota (10.71%), Thaumarchaeota (8.89%) and Alphaproteobacteria (8.05%).

Microbial Community Variation over Time

Individual variation in the 6 abundant groups at the 7 m depth was examined across all time points (Figure 5). *Actinobacteria* abundance at this depth ranged from 2% to 45% (median 27%). *Gammaproteobacteria* abundance was measured with a median of 17% with majority of the abundances concentrated on the low end of the scale. *Cyanobacteria* abundance was measured with a range from 5% to 31% (median of 15%).

For the *Euryarchaeota* present at the 7 m depth, the median abundance was measured to be 9% with a range from 2% to 18%. *Bacteroidetes* and *Alphaproteobacteria* abundances were measured with a median abundance of 7% and 6% respectively, while both groups had a majority of the abundances concentrated on the high end of the scale for that specific group.

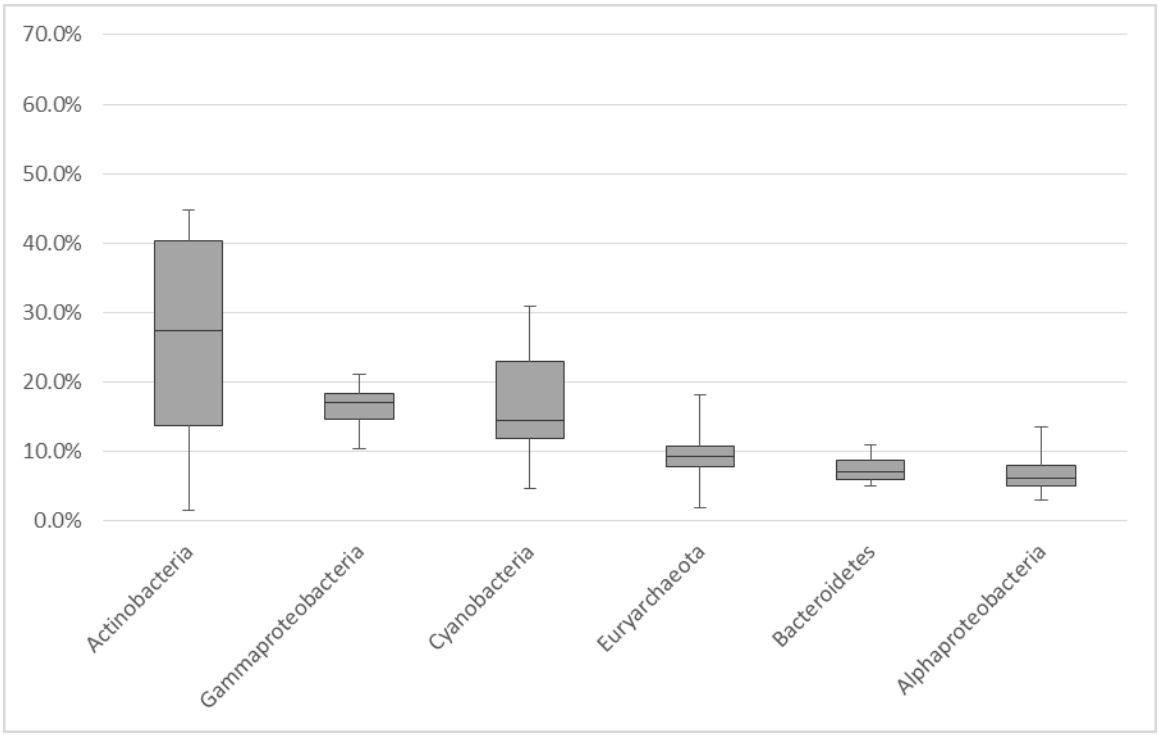

Figure 5: Variation in the 6 abundant (>1%) groups at the 7 m depth. Variation in the relative abundance of each abundant group across all time points was examined using standard box plots. The band inside each box is the median, while the bottom box is the first quartile and the top box is the third quartile. The ends of the whiskers represent the minimum and maximum relative abundances of each group. This graph depicts dispersion of an individual groups' relative abundances across all time points.

Variation in the 9 abundant groups at the 15 m depth was also examined (Figure 6). *Gammaproteobacteria* abundance was measured in a range from 9% to 27% (median 19%). *Cyanobacteria* abundance was measured with a median abundance of 14% and minimum and maximum abundances of 2% and 43% respectively. *Actinobacteria* present

at the 15 m depth, showed an abundance in the range from 4% to 62% (median abundance of 10%). *Euryarchaeota* abundance was measured in a range from 5% to 26% (median =11%). *Thaumarchaeota*, median abundance was measured to be 5% with a range from 1% to 33%. *Alphaproteobacteria* and *Deltaproteobacteria* abundances were measured with a median of 8% and 5% respectively while the minimum abundance of each group was 4% and 1% and the maximum abundance of each group was 15% and 9%. *Bacteroidetes* abundance at the 15 m depth was measured in a range from 3% to 12% (median=8%). *SAR406* present at this depth measured a 1% minimum abundance and an 8% maximum abundance with a median abundance of 4%.

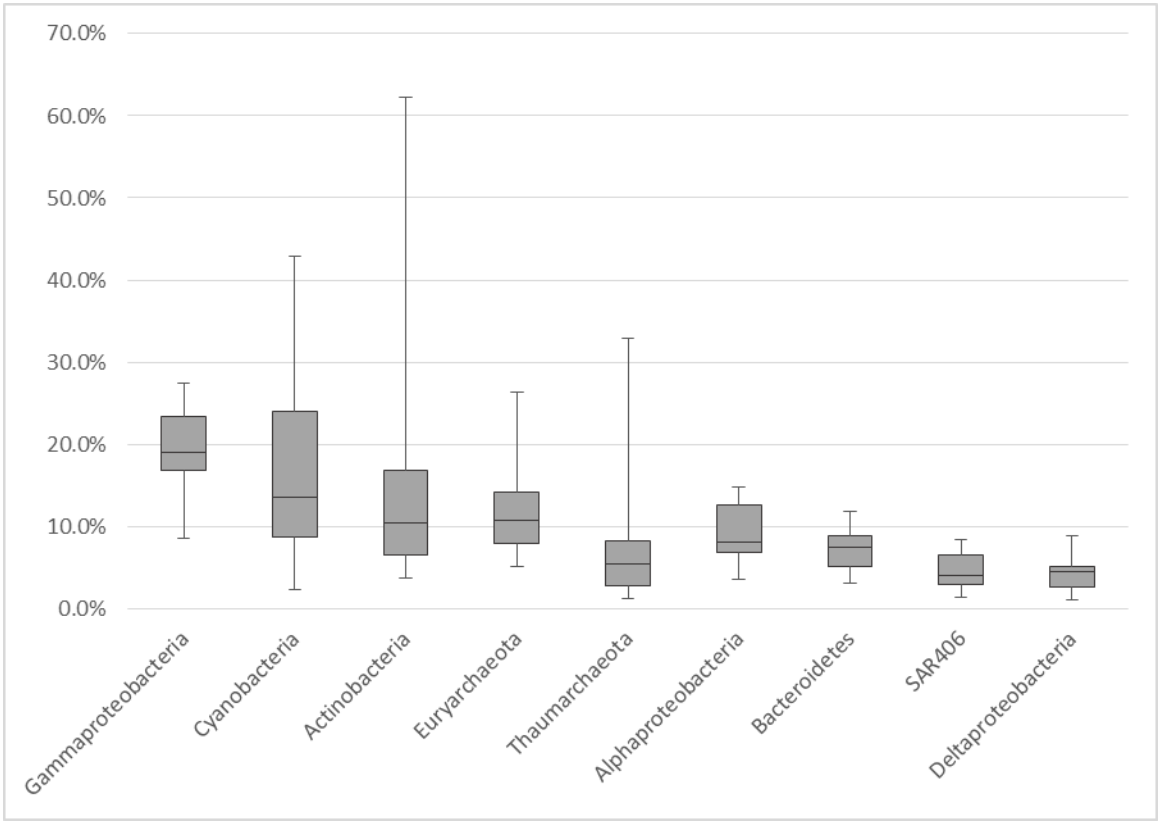

Figure 6: Variation in the 9 abundant (>1%) groups at the 15 m depth. Variation in the relative abundance of each abundant group was measured across all time points using standard box plots. The band inside each box is the median, while the bottom box is the first quartile and the top box is the third quartile. The ends of the whiskers represent the minimum and maximum relative abundances of each group. This graph depicts dispersion of an individual groups' relative abundances across all time points.

To examine the difference between microbial communities when comparing the 7 m depth to the 15 m depth, each groups' abundances across all time points at each depth were compared. Of the 29 major phyla present at this site, 7 groups were considered significantly different ($p<0.05$) when comparing the relative abundances at the 7 m depth to the relative abundances at the 15 m depth (Table 3). OTUs defined at the phylum level to be *Chlamydiae* showed a significant difference when measuring abundances at the 7m depth to the 15 m depth (n=10, p=0.0335, t test). Abundances of *Gemmatimonadetes* measured across all time points at the 7m depth versus the 15 m depth showed a significant difference as well (n=10, p=0.0427, t test). *Alphaproteobacteria* (n=10, p=0.0204, t test) and *Deltaproteobacteria* (n=10, p=0.0308, t test) also showed a significant difference in individual abundances when comparing the 7 m depth to the 15 m depth. OTUs representing *SAR406* also showed significant difference in relative abundance at each depth (n=10, p=0.0109, t test). *SBR1093* (n=10, p=0.0314, t test) and *Tenericutes* (n=10, p=0.0143, t test) showed a significant difference in relative abundance going from the 7 m depth to the 15 m depth as well.

OTUs	7m Mean Relative Abundance	15m Mean Relative Abundance	p-value	Observations
Chlamydiae	<1%	<1%	0.034*	10
Gemmatimonadetes	<1%	<1%	0.043*	10
Alphaproteobacteria	6.7%	9.0%	0.020*	10
Deltaproteobacteria	2.4%	4.1%	0.031*	10
SAR406	2.8%	4.3%	0.011*	10
SBR1093	<1%	<1%	0.031*	10
Tenericutes	<1%	<1%	0.014*	10

Table 3: Significant changes in the relative abundance of individual OTUs comparing the 7 m depth to the 15 m depth across all time points. To examine the difference between microbial communities when comparing the 7 m depth to the 15 m depth, each individual groups' abundances across all time points at each specific depth

were compared. 7 groups show significant changes between relative abundances between the 7 m and 15 m depths

CHAPTER 4

DISCUSSION

Marine microbial communities show high biodiversity and play predominant roles in many biogeochemical cycles on a global scale. Microbial communities in marine OMZs are structured by chemical, physical, and ecological parameters that changes dramatically along the vertical oxycline and into the OMZ. This study focused on assessing the microbial community's composition and structure between 2 varying depths over a diel cycle in the seasonal OMZ on the Louisiana Shelf in the northern Gulf of Mexico.

When considering both depths and all time points, the most dominate microbial phyla found at this site are consistent with microbial phyla identified in other marine coastal regions. The majority of sequences were affiliated with *Actinobacteria* (20.27%). The average relative abundance of *Actinobacteria* experienced an increase from the 15 m to 7 m depth, however, when comparing the relative abundance of *Actinobacteria* at the 7 m depth to the 15 m depth across all time points, there was not a significant change.

Two major Archaeal phyla, the *Euryarchaeota* and *Thamarchaeota,* dominated both depths, accounting for 19.6% of the total sequences found. Recent studies have shown the ubiquity as well as the biogeochemical importance of marine Archaea. However, the ecological parameters that control these communities are poorly understood (6). In our study, OTUs representing the *Euryarchaeota* phylum were abundant at both depths across all time points. At the class level, the *Euryacrchaeota* were assigned to the Thermoplasmata archaeal class. Indeed, this class has been identified in recent genomic studies examining active archaeal communities in marine surface water (11). The

majority of Thermoplasmata sequences were affiliated to the Marine Group II. Sequences matching members of the nitrifying *Thaumarchaeota* were also abundant in our dataset. The majority of *Thaumarchaeota* sequences were affiliated with the Genus *Nitrosopumilus* genus. *Nitrosopumilus* are autotrophs that oxidize ammonia to nitrite and therefore, members of this group, and other diverse *Thaumarchaeota* clades, are ubiquitous throughout the global ocean, notably along the periphery of marine low oxygen zones where they appear adapted to life under relatively low oxygen and ammonia concentrations. Due to their ubiquity, the *Thaumarchaeota* are now recognized as major players in the marine nitrogen cycle on a global scale (11). In recent studies in which examined the prevalence of ammonia-oxidizing archaea in the Gulf of Mexico revealed robust distributions of *Thaumarchaeota* at a coastal site with increasing abundances measured as depth increased (17). Relative abundances also positively correlated with stations near the Mississippi River Plume, suggesting and influence of riverine nutrients on ammonia oxidizing archaea (17). It has been suggested that members of Marine Group II are more abundant in temperate sea water than *Thaumarchaeota*, which is consistent with the microbial community at this station (6, 11).

OTUs representing *Cyanobacteria* were abundant across all time points at both the 7 m and 15 m depth. The average relative abundance of *Cyanobacteria* showed an increase from the 7m to the 15 m depth. However, when comparing the relative abundance of *Cyanobacteria* at each individual depth across all time points, there was not a significant change. Indeed, this is as expected due to the fact that both the 7 m and 15 m depths occurred in the photic zone. Overall, *Cyanobacteria* represented the third highest

average abundance when both depths were considered together with 16.91% of total sequences. When considered at the genus level, majority of the *Cyanobacteria* sequences were *Prochlorococcus*. These bacteria are photoautotrophs and are considered some of the most highly abundant marine microbes in surface waters, therefore, their abundance in the Gulf of Mexico OMZ is consistent with their ecophysiology (7).

In this study, *Gammaproteobacteria* and *Alphaproteobacteria* were also abundant across all time points at both the 7 m and 15 m depths. Overall, *Gammaproteobacteria* represented 17.85% of the total sequences found at station 6. The majority of *Gammaproteobacteria* were affiliated with the *Oceanospirillales* order. The average abundance of *Alphaproteobacteria* showed an increase of average relative abundance from the 7m depth to the 15 m depth. *Alphaproteobacteria* was also one of the 7 groups that showed a significant difference when comparing the relative abundances at both the 7 m and 15 m depths across all time points.

When considered at the genus level, majority of the *Alphaproteobacteria* were *Pelagibacter*. *Pelagibacter* are globally distributed aerobic heterotrophs that are estimated to make up ~25% of all planktonic marine bacteria (7). They are considered oligotrophs that grow slowly on low levels on nutrients. Indeed, due to the decrease in oxygen concentration and decrease in niche availability, it is consistent to see a significant difference in relative abundance of this bacteria going from the 7 m to the 15 m depth as the water column transitions into the core of the OMZ (16).

OTUs representing *Betaproteobacteria* went from being rare across all time points at the 7 m depth to cycling between rare and abundant at the 15 m depth. Also, when considering the 15 m depth, *Betaproteobacteria* was one of the groups that showed

a significant change in relative abundance when considering the relative abundances during the photic time points compared to the dark time points. The majority of the *Betaproteobacteria* sequences could not be classified past the subclass level. *Betaproteobacteria* have been shown to play a role in the marine nitrogen cycle as this group includes ammonia oxidizing bacteria, contributing to NH_4+ removal and NO_2- and N_2O production (10, 12). Therefore, co-occurrence of ammonia-oxidizing bacteria (*Beta-* and *Gammaproteobacteria*) combined with ammonia-oxidizing archaea (*Thaumarchaeota*) could help explain the nitrite and nitrate concentrations peaking at 6 μM at the 15 m depth, as well as the increase in ammonia oxidation rates at the 15 m depth, which were measured at 500 nmol/day.

OTUs representing the *Deltaproteobacteria* subclass went from being mostly abundant at the 7 m depth to being abundant at the 15 m depth. The average relative abundance increased from the 7 m depth to the 15 m depth going from 2.36% to 4.39% of all sequences. Also, *Deltaproteobacteria* was one of the 7 groups that showed a significant difference when comparing the relative abundances at both the 7 m and 15 m depths across all time points. Indeed, sulfate-reducing *Deltaproteobacteria* have been shown to be prevalent in permanent OMZs in waters below the oxycline (4). Sulfate-reducing bacteria are obligate anaerobes, therefore, it is consistent to see an increase in the overall averaged relative abundance moving into the core of the hypoxic zone at the 15 m depth. Subsequently, a positive correlation between abundances of sulfate-reducing *Deltaproteobacteria* and ammonia-oxidizing *Beta-* and *Gammaproteobacteria* as well as ammonia-oxidizing archaea has been recognized in permanent OMZs such as the ETNP (13). Therefore, to see the same positive correlation between these groups going from the

7 m depth to the 15 m depth, shows that similar microniches are in existence at this seasonal OMZ compared to permanent OMZs even though the size, depth, and water-circulation patterns of the two zones are highly different.

Overall, this site represents a highly variable marine ecosystem in which regional water mass circulation patterns shape the ecological parameters that control the system. The surface water is highly influenced by the influx of high nutrient, low salinity fresh water coming from the Mississippi and Atchafalaya Rivers (17). The microbial community present at this depth is shaped by a complex combination of environmental factors including the freshwater, high-nutrient mat that mixes with the warm coastal waters. High variability in the microbial community over the three day period of this study shows how horizontal spatial differentiation through water mass circulation possibly causes high rates of microbial community dispersion. This causes a unique, seasonal OMZ that is dominated by a unique microbial community that is altering an entire geologically important coastal region during the summer months.

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